CGRP, PACAP, and VIP Modulate Langerhans Cell Function by Inhibiting NF-κB Activation

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The neuropeptides calcitonin gene-related peptide (CGRP), pituitary adenylate cyclase-activating polypeptide (PACAP), and vasoactive intestinal peptide (VIP) suppress Langerhans cell (LC) antigen presentation and modulate cytokine production. We have tested the hypothesis that these neuropeptides (NP) inhibit LC function by modulating activation of NF-κB. Lipopolysaccharide (LPS) activates NF-κB in both a LC-like cell line (XS52) and epidermal LC enriched to ~95% and this effect is inhibited by each of the NP. Furthermore, CGRP, PACAP, and VIP suppress phosphorylation of IκB kinase β (P-IKKβ), prevent degradation of the IκBα, and inhibit activation of NF-κB. Thus, these NP modulate LC function by reducing NF-κB activation. Bay 11-7085, an inhibitor of IKK, reduced tumor necrosis factor-α (TNFα) production from LPS-stimulated XS52 cells and inhibited the ability of LC to present antigen to a T-cell clone *in vitro*. Each NP also inhibited LPS-induced secretion of TNFα by XS52 cells and LC enriched to ~95% homogeneity. We suggest that the inhibitory activities of CGRP, PACAP, and VIP on LC function are mediated, at least in part, by inhibition of P-IKKβ, which prevents IκBα degradation and activation of NF-κB. Modulation of this signaling pathway may be useful for therapeutic modulation of immunity in the skin.

Journal of Investigative Dermatology (2007) **127,** 2357–2367; doi:10.1038/sj.jid.5700858; published online 10 May 2007

INTRODUCTION

Langerhans cells (LC)[3] are professional antigen-presenting cells (APC) within the epidermis (Schmitt, 1999). They remain in a relatively immature state until they are activated through a series of events initiated by contact with an antigen. Until recently, it was believed that, once activated, these cells matured, took up and processed antigen, and migrated to regional lymph nodes where they presented the processed antigen to naive T cells, thereby initiating primary immune responses (Steinman *et al.*, 1995). Recently, a report utilizing conditional *langerin* knockout mice suggested that epidermal LC may actually downregulate the induction of cutaneous immunity (Kaplan *et al.*, 2005). Although this possibility remains controversial, our previously reported observations that epidermal nerves lie in close proximity to LC (Hosoi *et al.*, 1993) might suggest that release of inhibitory neuropeptides (NP) by those nerves could prevent LC from serving as effective APC (at least for T helper 1 responses) and contribute to cutaneous immune regulation.

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Abbreviations: APC, antigen-presenting cell; AU, arbitrary units; CGRP, calcitonin gene-related peptide; CHS, contact hypersensitivity; CM, complete medium; IKK, IκB kinase; KLH, keyhole limpet hemocyanin; LC, Langerhans cell; NP, neuropeptide; PACAP, pituitary adenylate cyclase-activating polypeptide; pLC, purified LC; VIP, vasoactive intestinal peptide

Received 30 October 2006; revised 1 March 2007; accepted 5 March 2007; published online 10 May 2007

Vasoactive intestinal peptide (VIP) and the related peptide pituitary adenylate cyclase-activating polypeptide (PACAP) are, respectively, 28- and 38-amino acid peptides. They are members of a superfamily of NP that includes secretin, glucagon, and growth hormone-releasing hormone and bind to an overlapping group of receptors. Two of these, VIP receptor type 1 and VIP receptor type 2, bind PACAP, and VIP with equal affinity. They are both G protein-coupled receptors that activate adenylate cyclase with consequent stimulation of cAMP production (Sherwood *et al.*, 2000; Laburthe *et al.*, 2002). VIP, PACAP and their receptors have been found in multiple organs, including the endocrine system, the reproductive system, and the immune system (Schulze *et al.*, 1997; Odum *et al.*, 1998; Mulder *et al.*, 1999). Studies have shown that PACAP and VIP have a predominantly immunosuppressive effect on peritoneal macrophages through regulation of cytokine production and NF-κB activation (Delgado *et al.*, 1999). Recently, we demonstrated that treatment of epidermal cells with either VIP or PACAP inhibited their ability to present antigen for elicitation of delayed-type hypersensitivity in previously immunized mice and intradermal administration of PACAP inhibited the induction of contact hypersensitivity (CHS) at the injected site. They also inhibited the ability of LC to present antigen to a T helper 1 clone and augmented the production of IL-10 by a lipopolysaccharide (LPS)-stimulated LC-like dendritic cell line, while downregulating LPS-stimulated IL-1β and IL-12 p40 production (Kodali *et al.*, 2003; Kodali *et al.*, 2004).

Another particularly interesting peptide is calcitonin gene-related peptide (CGRP), a 37-amino-acid peptide, widely

distributed in multiple organs including the central and peripheral nervous system and immune systems (Zaidi *et al.*, 1990). CGRP is co-expressed with ether somatostatin or substance P in sensory neurons (Brain and Williams, 1988) and is one of the major NP of the skin. We have reported that treatment *in vitro* with CGRP inhibited the ability of LC to stimulate allogeneic T cell and to present antigen to a T-cell hybridoma *in vitro* (Hosoi *et al.*, 1993), modulated LC cytokine production and suppressed LC maturation (Torii *et al.*, 1997). *In vitro* treatment of epidermal cells also inhibited their ability to present antigen for immunization of mice by subcutaneous injection and intradermal administration of CGRP inhibited the induction of immunity to a hapten painted at the injection (Asahina *et al.*, 1995).

A more complete understanding of the molecular mechanisms through which PACAP, VIP, and CGRP attenuate LC immune function would help explain how the nervous system influences cutaneous immunity and may illuminate new therapeutic approaches to the treatment of inflammatory skin disorders. In this regard, the NF-κB transcription factor system is of particular importance. In unstimulated cells, NF-κB exists in an inactive state in the cytoplasm complexed with the inhibitory protein IκB (Viatour *et al.*, 2005). Upon cell activation by various stimuli, including LPS and cytokine stimulation, IκB kinase (IKK) becomes activated. IκBα then undergoes phosphorylation and degradation and the NF-κB heterodimer is translocated into the nucleus where it binds to DNA and activates transcription (Li and Verma, 2002). A role for NF-κB in various aspects of the immunobiology of APC has been reported. NF-κB activation appears to be extremely important in determining the functional development of APC (Thomas *et al.*, 2005). Inflammatory stimuli such as LPS, tumor necrosis factor-α (TNFα), and IL-1 induce DC maturation through the NF-κB pathway (Ardeshna *et al.*, 2000). The NF-κB family of transcription factors has multiple roles in regulating events associated with differentiation of DCs and presentation of antigen to T cells in lymphoid tissue (Pettit *et al.*, 1997; Ghosh *et al.*, 1998), APC can mature via an alternative NF-κB activation path independent of IκBα degradation (Thomas *et al.*, 2005). Importantly, elevated NF-κB activation results in overall enhanced APC antigen presentation function (Poligone *et al.*, 2002; Sen *et al.*, 2003). Taken together, NF-κB activation appears to play a significant role in stimulating antigen presentation. As NF-κB plays a central role in regulating the transcription of proinflammatory cytokines and co-stimulatory molecules (including CD80 and CD86) important for antigen presentation (Rescigno *et al.*, 1998; Jeon *et al.*, 1999; Feldmann *et al.*, 2002), we asked whether PACAP, VIP, or CGRP could regulate the activation of this transcription factor. Furthermore, as production of TNFα is tightly linked to NF-κB (Trede *et al.*, 1995; Yao *et al.*, 1997), we also examined the influence of these NP on induction of TNFα secretion as, if these NP inhibit NF-κB activation, they should also inhibit induction of TNFα secretion. The results of this study indicate that PACAP, VIP, and CGRP suppress phosphorylation of IκB kinase *β* (P-IKK*β*), prevent degradation of IκBα, and thus inhibit activation of NF-κB. They also inhibit the induction of TNFα secretion. These effects were observed in both a LC-like cell line and fresh LC enriched to 95%.

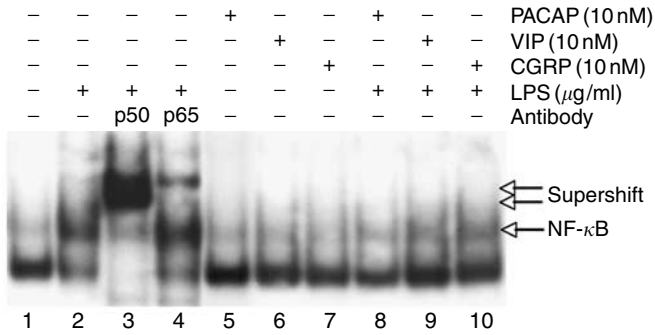
RESULTS

PACAP, VIP, and CGRP inhibit LPS-induced NF-κB DNA binding in XS52 cells

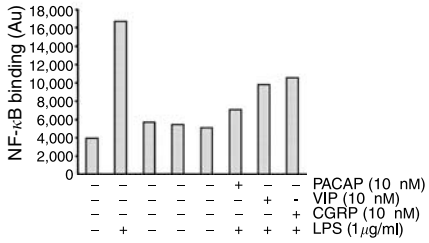
NF-κB is a transcriptional regulator involved in the expression of co-stimulatory molecules and cytokines that facilitate antigen presentation (Ardeshna *et al.*, 2000; Rescigno *et al.*, 1998; Jeon *et al.*, 1999; Feldmann *et al.*, 2002). To test whether the inhibitory effects of PACAP, VIP, and CGRP on LC function might be mediated by NF-κB, we examined the effect of these NP on LPS-stimulated NF-κB activation using an electrophoretic mobility shift assay. XS52 cells were cultured in the presence or absence of PACAP, VIP, or CGRP for 1 hour and then LPS was added to some cultures for 3 hours to activate NF-κB. LPS caused a significant increase in NF-κB activation (Figure 1a, lanes 1 *vs* 2), and this band was shifted by incubation with antibody (Ab) to p50 or p65 (Figure 1a, lanes 3 and 4), demonstrating that we were measuring the authentic transcription factor. PACAP, VIP, and CGRP each inhibited the LPS-induced NF-κB activation (Figure 1a, compare lane 2 with lanes 8, 9, and 10). To obtain a quantitative measure of the degree of suppression observed, the gel from this experiment and two others were analyzed by densitometry. The data in the table in Figure 1a show the degree of suppression observed for each NP in these three experiments. Thus, these NP inhibit NF-κB activation. To begin to determine the site of this inhibition, we measured the effect of the NP on the degradation of IκBα and the activation of IKK*β*.

Figure 1. PACAP, VIP, and CGRP inhibit LPS-induced P-IKK*β* preventing IκB degradation and subsequent NF-κB nuclear translocation in XS52 and pLC. (**a**) PACAP, VIP, and CGRP inhibit NF-κB DNA binding. XS52 cells were stimulated for 1 hour in the presence or absence of PACAP, VIP, or CGRP (10^{-8} M) followed by addition of 1 *μ*g/ml of LPS to some culture wells for 3 hours. Nuclear extracts were then prepared, probed, and used in the electrophoretic mobility shift assay. For supershifting, nuclear extracts were incubated with polyclonal Abs against p50 or p65 for 10 minutes before the addition of the NF-κB probe. The bar graph shows the density of the bands in the gel shown and the table shows the percent inhibition in each of three experiments. (**b**) PACAP, VIP, and CGRP prevent degradation of IκBα in XS52 cells. XS52 cells were stimulated with 1 *μ*g/ml of LPS with or without the presence of PACAP, VIP, or CGRP (10^{-8} M). The cytosolic amounts of IκBα at different time points were determined by immunoblot; equal amounts of protein were used to detect glyceraldehyde-3-phosphate dehydrogenase as a control (lower panel). The bar graph shows the relative density of the bands in the gel shown and the table shows the percent of IκBα degradation of three experiments. (**c**) PACAP, VIP, and CGRP reduced LPS-induced degradation of IκB in pLC. Fresh pLC from BALB/c mice were stimulated for 30 minutes with or without 1 *μ*g/ml of LPS in the presence or absence PACAP, VIP, or CGRP (10^{-8} M). The cytosolic amounts of IκBα were detected by immunoblotting; equal amounts of protein were used to detect IKK*β* to insure equivalent loading of protein (lower panel). The bar graph shows the relative density of the bands in the gel shown and the table shows the percent of IκBα degradation of three experiments. (**a**, **b**, and **c**) One representative experiment of three performed is presented.

a



% of NF-κB inhibition			
	LPS+PACAP	LPS+VIP	LPS+CGRP
Exp#1	89	66	57
Exp#2	85	64	45
Exp#3	63	67	49



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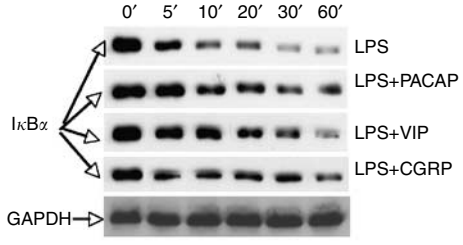
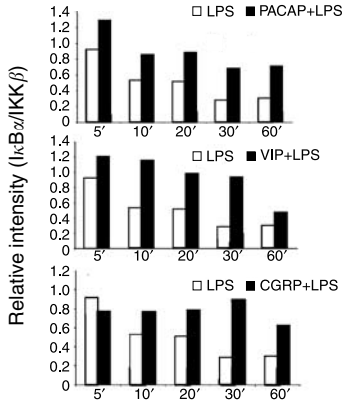
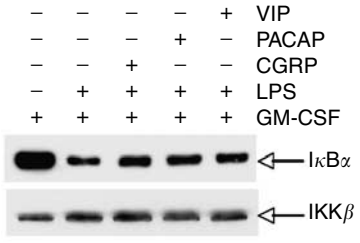
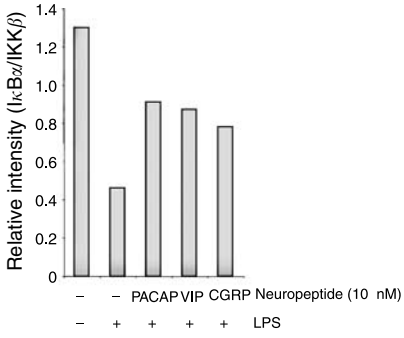




% IκBα degradation															
	Exp#1					Exp#2					Exp#3				
Min	5′	10′	20′	30′	60′	5′	10′	20′	30′	60′	5′	10′	20′	30′	60′
LPS	42	68	70	82	81	30	39	49	69	76	12	19	34	69	71
LPS+PACAP	15	44	43	56	54	10	14	21	44	49	0	8	10	13	11
LPS+VIP	30	42	40	59	53	10	11	21	49	59	0	8	12	45	35
LPS+CGRP	28	30	40	44	62	15	13	14	39	69	10	15	23	22	33

c





% IκBα degradation				
	LPS	LPS+PACAP	LPS+VIP	LPS+CGRP
Exp#1	70	35	34	37
Exp#2	82	45	55	58
Exp#3	70	47	48	43

PACAP, VIP, and CGRP inhibit NF-κB nuclear translocation by reducing LPS-induced degradation of IκBα in XS52 cells and LC

NF-κB activation is mediated by the phosphorylation and degradation of IκBα (Viatour *et al.*, 2005). To determine whether PACAP, VIP, or CGRP prevent degradation of IκBα, we exposed XS52 cells to 1 μg/ml of LPS in the presence or absence of PACAP, VIP, or CGRP and measured IκBα by immunoblotting. As expected, IκBα was degraded after the cells were exposed to LPS stimulation (Figure 1b, top). PACAP, VIP, and CGRP each reduced the degradation of IκBα over time (5–60 minutes; Figure 1b). Glyceraldehyde-3-phosphate dehydrogenase was measured to insure equivalent loading of protein. In a separate experiment, in the absence of LPS, no significant change in expression of IκBα was observed over the time range examined (up to 60 minutes) (data not shown).

To test whether these NP had a similar effect on authentic LC, fresh purified LC (pLC) from BALB/c mice were cultured in the complete medium (CM) supplemented with 10 ng/ml of mGM-CSF for 1 hour. Some cultures were exposed to 1 μg/ml of LPS in either the presence or absence of PACAP, VIP, or CGRP (10 nM). Anti-IκBα immunoblots of cell extracts prepared at 30 minutes showed that the each of the NP reduced the LPS-induced degradation (Figure 1c). Thus, PACAP, VIP, and CGRP inhibit LPS-stimulated IκBα degradation in both authentic LC and LC-like lines.

PACAP, VIP, and CGRP inhibit P-IKKβ in XS52 cells and LC

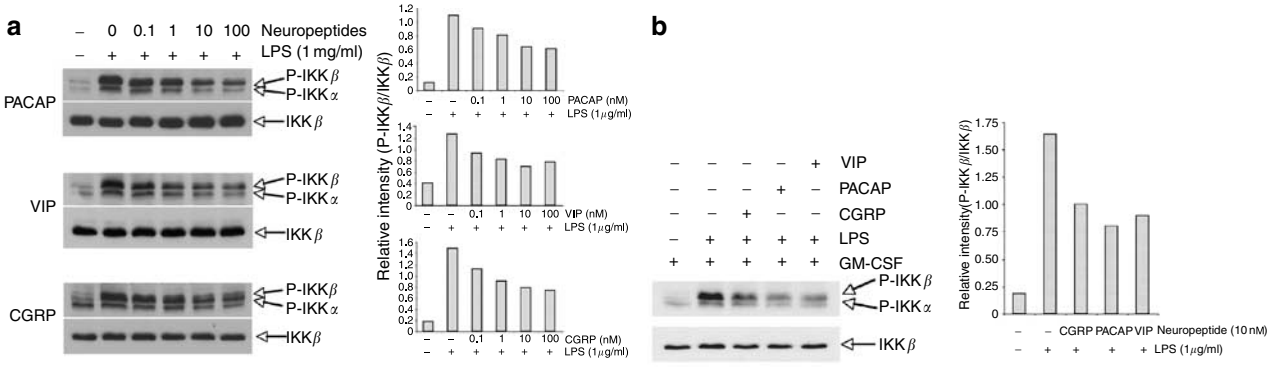
Phosphorylation and activation of IKK promotes phosphorylation and degradation of IκB (Viatour *et al.*, 2005), so we next chose to determine if PACAP, VIP, or CGRP inhibit the activation of IKK in both XS52 and authentic LC. Cultures of XS52 cells (Figure 2a) and pLC (Figure 2b) were exposed to LPS in the presence or absence of PACAP, VIP, or CGRP for 30 minutes as indicated. Thirty minutes of stimulation was chosen as this yields the maximum expression of IKKα and IKKβ (data not shown). We measured P-IKKβ by immunoblotting using an Ab against the phosphorylated forms of IKKα/β. We measured the level of total IKKβ using an Ab that recognized IKKβ. LPS induced P-IKKβ (Figure 2a, lane 1 *vs* 2), whereas PACAP, VIP, and CGRP inhibit this P-IKKβ in a dose-dependent manner (Figure 2a, compare lane 2 with lanes 3, 4, 5, and 6). Likewise, each of the three NP reduced LPS-dependent P-IKKβ in authentic pLC (Figure 2b). None of these peptides influenced the absolute level of IKKβ. In one of three experiments, P-IKKα was inhibited by CGRP, PACAP, and VIP. In two of three experiments, this was not observed with densitometry. However, in these two experiments, the bands for IKKα were very light and it remains possible that these NP inhibit P-IKKα.

Bay 11-7085 inhibits LPS-induced P-IKKβ in XS52 cells

Bay 11-7085 is an irreversible inhibitor of IκB phosphorylation (Pierce *et al.*, 1997; Mabuchi *et al.*, 2004). We examined its ability to inhibit P-IKKβ. XS52 cells were exposed to 1 μg/ml of LPS in the absence or presence of increasing concentrations of Bay 11-7085 (0–10 μM) for 30 minutes. The cytosolic content of P-IKKβ was detected by immunoblotting. LPS induced P-IKKα/β (Figure 3, lane 1 *vs* 2), whereas Bay 11-7085 inhibited P-IKKβ in a dose-dependent manner (Figure 3, compare lane 2 with lanes 3, 4, 5, and 6). Thus, Bay 11-7085 inhibits P-IKKβ.

NF-κB participates in antigen presentation by epidermal LC to a T-cell clone

PACAP, VIP, and CGRP each inhibit antigen presentation by LC (Hosoi *et al.*, 1993; Kodali *et al.*, 2003; Kodali *et al.*,



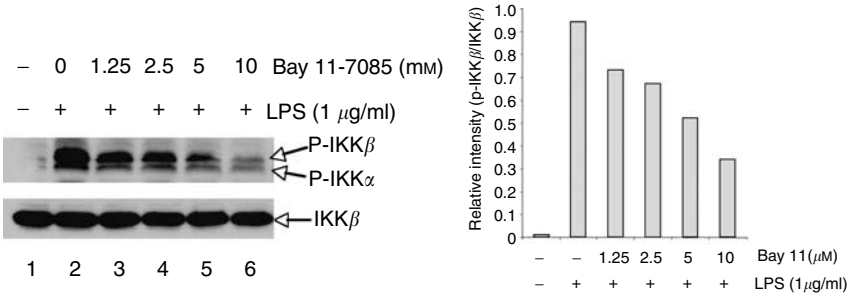
% of P-IKKβ inhibition

	LPS	LPS+PACAP				LPS+VIP				LPS+CGRP			
		0.1nM	1nM	10 nM	100 nM	0.1nM	1nM	10 nM	100 nM	0.1nM	1nM	10 nM	100 nM
Exp#1	0	21	30	48	51	39	53	68	59	28	44	54	58
Exp#2	0	16	42	59	60	30	38	50	50	25	48	52	51
Exp#3	0	9	27	40	48	24	33	49	60	11	11	25	32

% of P-IKKβ inhibition

	LPS	LPS+PACAP	LPS+VIP	LPS+CGRP
Exp#1	0	53	52	42
Exp#2	0	53	60	56
Exp#3	0	52	49	41

Figure 2. PACAP, VIP, and CGRP suppress P-IKKβ in both XS52 and freshly obtained epidermal LC. (**a**) PACAP, VIP, and CGRP suppressed P-IKKβ in XS52 cells. Cultures of XS52 cells were exposed to 1 μg/ml LPS in the presence or absence of PACAP, VIP, or CGRP (10^{-7}–10^{-11} M) for 30 minutes. Total protein was then extracted and the amount of P-IKKβ was determined by immunoblot using an Ab against both phosphorylated forms of IKK (P-IKKα and P-IKKβ). Equal amounts of protein were loaded into control wells to detect IKKβ (lower panel). (**b**) PACAP, VIP, and CGRP inhibited P-IKKβ in pLC. Fresh pLC from BALB/c mice were cultured in 10 ng/ml of mGM-CSF for 1 hour. Then, they were stimulated for 30 minutes with or without 1 μg/ml of LPS in the presence or absence of PACAP, VIP, and CGRP (10^{-8} M). Total protein was extracted and the amount of P-IKKβ was determined by immunoblot. Equal amounts of protein were loaded into control wells to detect IKKβ. The bar graph shows the relative density of the bands in the gels shown and the table shows the percent of inhibition of IKKβ phosphorylation in three experiments. (**a** and **b**) One representative experiment of three performed is presented.



% of P-IKKβ inhibition					
	LPS				
	Bay 11-7085 0 μM	Bay 11-7085 1.25 μM	Bay 11-7085 2.5 μM	Bay 11-7085 5 μM	Bay 11-7085 10 μM
Exp#1	0	18	28	41	62
Exp#2	0	37	52	76	79
Exp#3	0	30	46	58	85

Figure 3. Bay 11-7085 inhibited P-IKKβ by XS52 cells. XS52 cells were exposed to 1 μg/ml of LPS with or without the presence of Bay11-7085 (0–10 μM). The cytosolic content of P-IKKα/β was detected by immunoblotting and control wells were used to detect IKKβ to insure equivalent loading of protein (lower panel). The bar graph shows the relative density of the bands in the gel shown and the table shows the percent of inhibition of IKKβ phosphorylation in three experiments.

2004), but it is not clear whether NF-κB activation is important for antigen presentation by LC. To determine if activation of NF-κB contributes to antigen presentation by LC, we cultured epidermal cells (which are ~2% LC) in the absence or presence of increasing concentrations of Bay 11-7085, and then exposed them to keyhole limpet hemocyanin (KLH) in the presence of the same concentration of Bay 11-7085. Bay 11-7085 was carefully removed by multiple washings, and the ECs were co-cultured with the KLH-responsive T helper 1 clone, HDK-1, for 72 hours. We measured IFN-γ production by ELISA as an index of HDK-1 cell activation. Antigen-exposed ECs stimulated a significant increase in IFN-γ production, and this increase was inhibited by Bay 11-7085 (Figure 4). This inhibition occurred with an EC_{50} of about 0.6 μM, strongly suggesting that NF-κB activation is central to antigen presentation by LC. The ability of NP to inhibit NF-κB activation may be sufficient to explain their effects on both TNFα expression and antigen presentation.

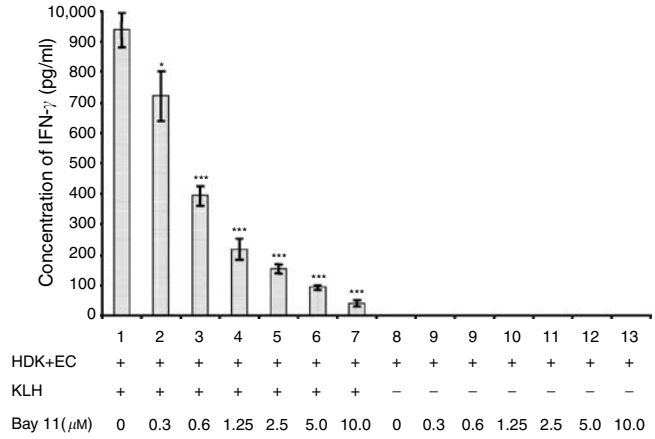


Figure 4. Bay 11-7085 inhibited the ability of epidermal LC to present antigen to a T-cell clone. Epidermal cells from BALB/c mice were preincubated in CM (bar 1) or different concentration of Bay 11-7085 (bars 2–7) for 1.5 hours and then exposed to KLH for an additional 1.5 hours. Negative controls (bars 8–13) were preincubated with or without different concentrations of Bay 11-7085 and were not treated with KLH. ECs were then washed and cocultured with cells of the KLH-specific clone HDK-1. Supernatants were collected at 72 hours and IFN-γ content assessed by ELISA. Each results is the mean ± SD of three separate experiments performed in duplicate. Previous experiments have shown no significant activation in the absence of APC or antigen. In the presence of antigen, but no APC, a small amount of response from HDK-1 cells is seen (<5% of the positive control in most experiments). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

NF-κB mediates LPS-dependent induction of TNFα

TNFα production is linked to NF-κB activation in several cell types. We wished to confirm that this is the case in XS52 cells. We inhibited NF-κB activation with Bay 11-7085. XS52 cells were exposed to LPS in the presence or absence of Bay 11-7085 for 4 hours, and the amounts of TNFα production in the culture supernatants were assayed by ELISA. LPS greatly enhanced TNFα production, but Bay 11-7085 inhibited LPS-induced TNFα production dose dependently (Figure 5a). In additional experiments performed in the same manner, supernatants were prepared from XS106 cells (chosen because they produce high levels of IL-12 p40 and IL-1β) and examined by ELISA for IL-12 p40 and IL-1β content. As shown by the data in Figure 5b, Bay 11-7085 also potently inhibited the induction of IL-12 p40 and IL-1β. As PACAP, VIP, and CGRP inhibit the activation of IKK and NF-κB activation, they should also inhibit the production of TNFα, so we next examined this hypothesis.

PACAP, VIP, and CGRP inhibit LPS-induced TNFα production in both XS52 cells and pLC

As production of TNFα is tightly linked to NF-κB activation, we next examined the effect of PACAP, VIP, and CGRP on TNFα secretion. XS52 cells were stimulated with different

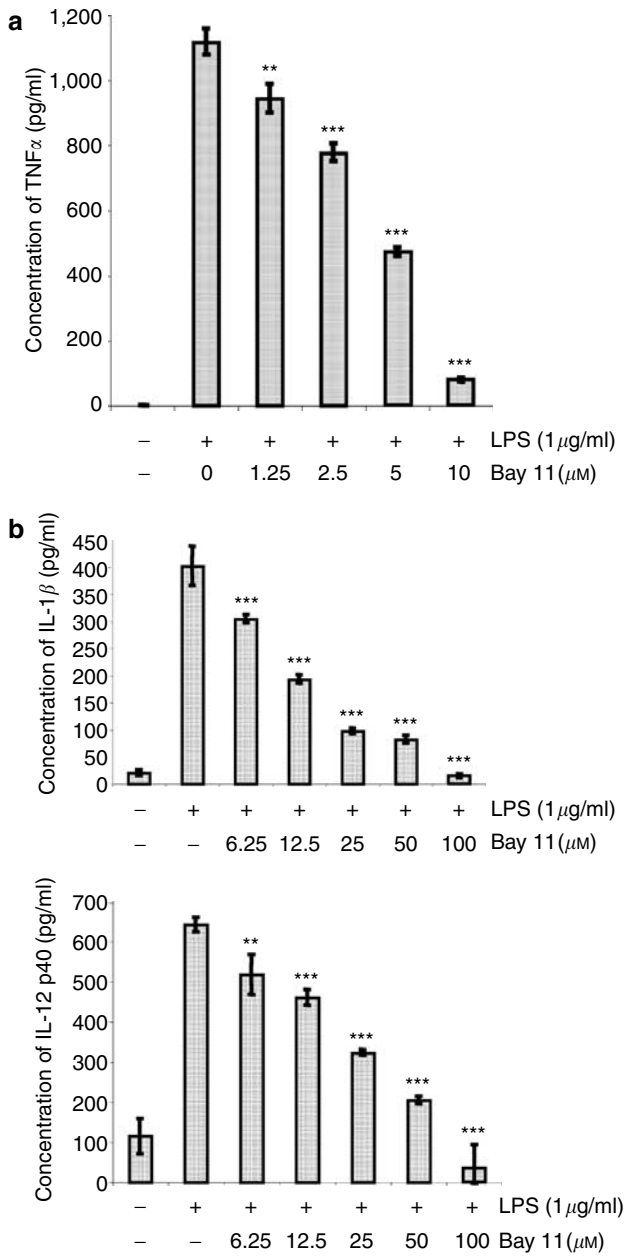


Figure 5. Bay 11-7085 inhibited TNFα, IL-12 p40, and IL-1β production by XS52 cells. (**a**) XS52 cells were stimulated with 1 μg/ml of LPS in the presence or absence of Bay 11-7985 (0–10 μM) for 4 hours. Supernatants were collected and TNFα release was determined by ELISA. The data shown represent the mean + SD of results obtained in an experiment performed in three separate plates prepared at the same time where each plate was used as a data point to compute the average value ($N = 3$). Two completely separate experiments were performed on separate days that yielded similar results. (**b**) In a separate experiment supernatants were assayed for IL-12 p40 and IL-1β. Each result represents the mean + SD of results obtained in an experiment performed in three separate plates prepared at the same time where plate was used as a data point to compute the average value ($N = 3$). A completely separate experiment was performed on a separate day that yielded similar results. $^{**}P < 0.01$, $^{***}P < 0.001$.

concentration of LPS in the absence or presence of various doses of PACAP, VIP, or CGRP. We assayed the TNFα content of culture supernatants by ELISA at different time periods. LPS induced the production of TNFα within 90 minutes, and PACAP, VIP, and CGRP each inhibited the induction of TNFα production at each time point tested (Figure 6a). Inhibition of TNFα production by PACAP, VIP, and CGRP was significant and dose-dependent over the concentration range of 1–100 nM (Figure 6b), with maximal effects at 10 nM (Figure 6b). PACAP, VIP, and CGRP inhibited TNFα production at all concentrations of LPS tested (0.01–1,000 ng/ml) (Figure 6c). Thus, PACAP, VIP, and CGRP inhibit the production of TNFα.

To determine whether NP also downregulate the induction of TNFα in authentic epidermal LC, we prepared pLC from BALB/c mice. Some cultures were exposed to LPS in the presence or absence of each NP. PACAP, VIP, and CGRP each inhibited LPS-induced TNFα production (Figure 7). We conclude that PACAP, VIP, and CGRP reduce the induction of TNFα by LPS in highly enriched preparations of LC. We suggest that the ability of PACAP, VIP, and CGRP to reduce the activation of IKK and NF-κB contributes significantly to their ability to reduce antigen presentation, the expression of TNFα, and, probably, other markers of LC maturation.

DISCUSSION

PACAP, VIP, and CGRP downregulate several aspects of LC immune function, including antigen presentation and the induction of IL-1β and IL-12 p40 (Hosoi *et al.*, 1993; Torii *et al.*, 1997; Kodali *et al.*, 2003, 2004). Indeed, suppression of antigen presentation by PACAP, VIP, and CGRP has been demonstrated in several different systems and models. Additionally, injection of CGRP or PACAP intradermally into mice inhibits the acquisition of CHS at the injected site.

As PACAP, VIP, and CGRP each inhibit antigen presentation by LC and inhibit the LPS-induced induction of IL-1β and IL-12 p40 production, we hypothesized that the effect of these agents on LC function may be through inhibition of activation of NF-κB. The results described above strongly support this hypothesis. Each agent suppressed phosphorylation of IKKβ and inhibited the degradation of IκBα, thus inhibiting activation of NF-κB. They also inhibit the induction of TNFα secretion. We also employed the pharmacologic inhibitor of NF-κB activation, Bay 11-7085, to determine its ability to inhibit TNFα production and LC antigen presentation function. This agent irreversibly inhibits the phosphorylation of IκB (Pierce *et al.*, 1997; Mabuchi *et al.*, 2004) and we found that it also inhibits the P-IKKβ. Bay 11-7085 inhibited the activity of IKK, disrupting the NF-κB/IκB regulatory pathway and decreased the ability of LC to present antigen to a T-cell clone. Bay 11-7085 also inhibited TNFα production from LPS-stimulated XS52 cells as well as IL-12 p40 and IL-1β production by XS106 cells. Without P-IKKα/β, the NF-κB dimer is not released and is unable to translocate into the nucleus where it can bind to promoter sites on DNA. Thus, this pharmacologic NF-κB inhibitor strikingly mimics the effects of PACAP, VIP, and CGRP in this system. The effects of these NP on LC appear to be mediated, at least in part, by inhibition of the NF-κB activation pathway. Three hours of exposure of fresh LC to concentrations of Bay 11-7085 below 10 μM did not affect viability 24 hours later as assessed by FACS analysis (data not shown).

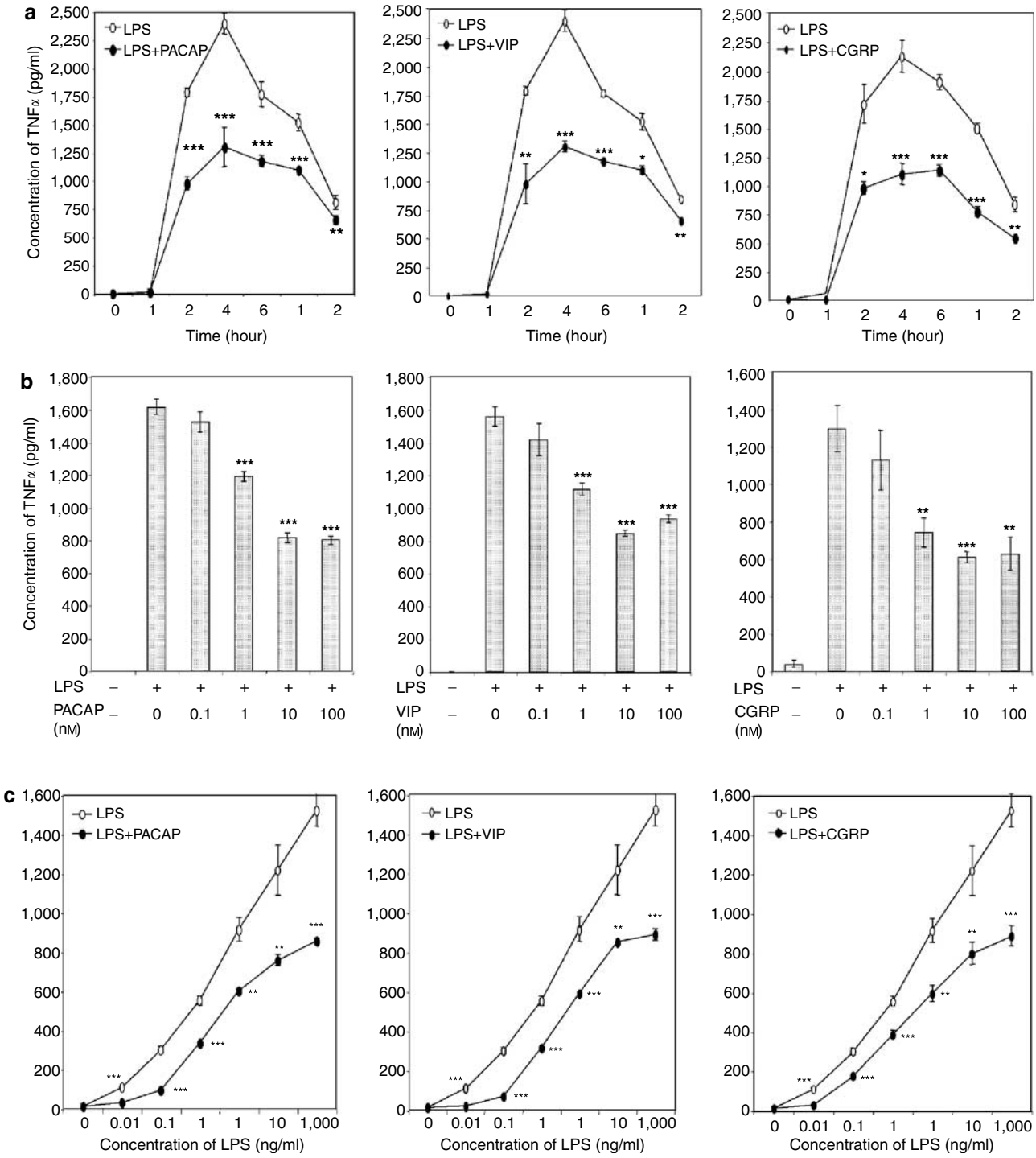


Figure 6. PACAP, VIP, and CGRP inhibited TNFα production by LPS-stimulated XS52 cells. (**a**) XS52 cells (2.5×10^5 cells/ml) were exposed to 0.1 μg/ml of LPS in the presence or absence of 10^{-8} M CGRP, PACAP, or VIP. Supernatants were collected at different times and assayed for TNFα production by ELISA. (**b**) Dose–response for the inhibitory effect of PACAP, VIP, and CGRP on LPS-stimulated TNFα production. XS52 cells (2.5×10^5 cells/ml) were exposed to 0.1 μg/ml of LPS in the presence of a range of concentration of PACAP, VIP, or CGRP (0–100 nM) for 4 hours. Supernatants were collected and assayed by ELISA for TNFα production. (**c**) PACAP, VIP, and CGRP inhibited TNFα production over a range of LPS concentrations (0.01–1,000 ng/ml). XS52 cells were stimulated with LPS in the presence or absence of 10^{-8} M of CGRP, PACAP, or VIP. Supernatants were collected after a 4 hour incubation period, and TNFα release was determined by ELISA. The data shown represent the mean + SD of results obtained in an experiment performed in three separate plates prepared at the same time where the average of wells in each plate was used as a data point to compute the average value. Two completely separate experiments were performed on separate days that yielded similar results. *$P<0.05$, **$P<0.01$, ***$P<0.001$ (**a**, **b**, and **c**).

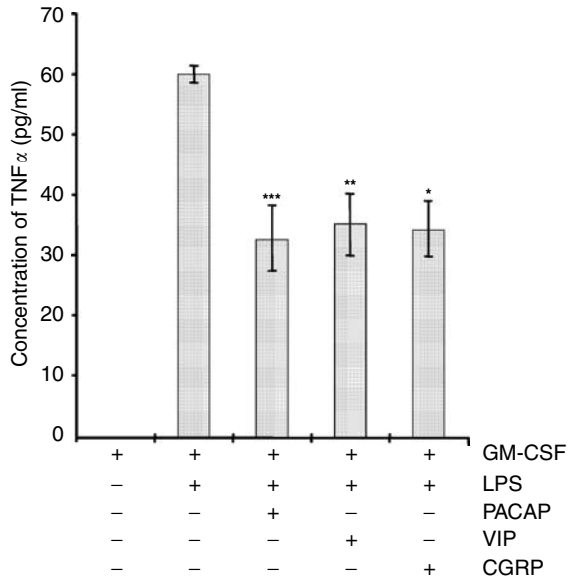


Figure 7. CGRP, PACAP, and VIP inhibited TNFα production by LPS-stimulated fresh pLC. Fresh pLC from BALB/c mice were cultured in CM containing 10 ng/ml of mGM-CSF for 1 hour and then stimulated with or without 0.1 μg/ml of LPS in the presence or absence of 10 nM CGRP, PACAP, or VIP. Supernatants were collected after 6 hours of stimulation and assayed for TNFα content by ELISA. The data shown represent the mean + SD of results obtained in an experiment performed in three separate plates prepared at the same time where the average of wells in each plate was used as a data point to compute the average value. Two completely separate experiments were performed on separate days that yielded similar results. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

PACAP, VIP, and CGRP clearly inhibited TNFα production by XS52 cells and pLC and transcriptional regulation of the TNFα gene is also known to be regulated by NF-κB (Trede *et al.*, 1995; Yao *et al.*, 1997). This is of considerable interest as TNFα plays an important role in the promotion of LC maturation (Caux *et al.*, 1992) and antigen presentation (Grabbe *et al.*, 1992). TNFα is also a stimulator for the migration of LC (Cumberbatch *et al.*, 1999; Cumberbatch *et al.*, 2003). During the sensitization phase of CHS, contact allergens stimulate epidermal cells to synthesize and release proinflammatory cytokines such as TNFα and IL-1β, which in turn promote LC migration from skin. Knockout mice lacking the p75 TNFα receptor exhibited depressed LC migration and reduced CHS response (Wang *et al.*, 1997).

However, there is some controversy regarding the ability of LC to produce TNFα. Although there are reports that highly enriched preparations of LC can make TNFα (Caux *et al.*, 1992, Fujita *et al.*, 2005), Schreiber *et al.* (1992) stated that LC likely do not produce significant amounts of TNFα and that apparent TNFα production in highly enriched LC populations is probably coming from contaminating keratinocytes. However, other investigators have reported that dendritic LC generated from human cord blood produced TNFα, both spontaneously and after stimulation with CD40 (de Saint-Vis *et al.*, 1998). Our results indicate that PACAP, VIP, and CGRP inhibited LPS-induced TNFα production in a dose-dependent manner in both XS52 cells and 95% LC from 10^{-7} to 10^{-10} M, with the maximum effect being observed at 10^{-8} M. As XS52 is a cell line, the effect would seem to be certain in that system. We cannot exclude the possibility that with a 95% LC population, production of TNFα is actually from a few contaminating keratinocytes with the locus of inhibitory activity of the NP for TNFα at the keratinocyte. We also recognize that as XS52 is a transformed dendritic cell line, its ability to produce TNFα may be quite different from that of LC *in situ*. Additionally, it is possible that the cell biology of LC may be altered by the procedures needed to isolate them.

The ability of NP to inhibit NF-κB activation may explain, at least in part, their effects on both cytokine expression and antigen presentation. Additionally, the recent report that LC may downregulate hypersensitivity in the skin may have relevance to our findings. Kaplan *et al.* (2005) recently reported on transgenic mice in which the regulatory elements from human langerin were used to drive expression of diphtheria toxin. These mice lacked epidermal LC but were otherwise intact. It was found that these mice had enhanced CHS responses and that the LC exerted their regulatory effect in the priming phase of CHS (Kaplan *et al.*, 2005). Two other reports of mice in which the diphtheria toxin receptor gene is ''knocked-in'' to the *langerin* locus are relevant to this discussion (Bennett *et al.*, 2005; Kissenpfennig *et al.*, 2005). In this model, injection of mice with diphtheria toxin leads to a loss of langerin^{+} cells. In one of these reports, a significant decrease in CHS was observed with loss of LC (Bennett *et al.*, 2005), whereas in the other (Kissenpfennig *et al.*, 2005), there was no significant change in the CHS reponse. The reasons for these discordant results are not clear, although Kaplan *et al.* (2005) speculate that it may relate to the sparing of spleen and lymph node langerin^{+} cells with their technique. If it is true that LC are downregulatory for CHS response in the skin, we speculate that, as many LC are in close approximation with epidermal nerves (Hosoi *et al.*, 1993), nerve-derived NP may contribute to this phenomenon by keeping LC in a relatively immature state.

A more complete understanding of how NP such as PACAP, VIP, and CGRP regulate LC function may reveal insights that lead to novel approaches to the treatment of skin disorders characterized by abnormal immune reactivity. Furthermore, if the conditions of the release of these factors were regulated by psychologic state, these findings may help to explain how psychologic state could influence cutaneous immunity.

MATERIALS AND METHODS

Mice

Female BALB/c (H-2^{d}) mice (6- to 12-week-old) were purchased from the Jackson Laboratory (Bar Harbor, ME). The mice were kept in the animal facility of Weill Medical College of Cornell University on a 12 hour light/dark cycle. All experiments using mice were reviewed and approved by the Weill Cornell Institutional Animal Care and Use Committee.

Reagents

Rat PACAP$_{6-38}$, VIP, and CGRP were purchased from Peninsula Laboratories (San Carlos, CA); LPS and Bay11 7085 was purchased

from Sigma-Aldrich (Saint Louis, MO); rabbit monoclonal anti-IκBα and anti-phosphorylated IKKα/IKKβ Ab were purchased from Cell Signaling Technology (Beverly, MA); anti-p50 and anti-p65 Ab were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell line and cell culture

CM consisted of Roswell Park Memorial Institute 1640 medium (Cellgro, Herndon, VA) containing 10% fetal calf serum (Gemini Bio-Products, Woodland, CA), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1 mM nonessential amino acids, 0.1 mM essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, and 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid buffer (all CM components were purchased from Mediatech (Herndon, VA).

For preparation of conditioned supernatants, cells were cultured in CM with addition of 2 μl of phosphate-buffered saline (PBS) containing a NP and the same volume of PBS was added to each control well.

The XS52 and XS106 cell lines were generously supplied by A Takashima (University of Texas, Southwestern Medical Center, Dallas, TX). They are LC-like lines derived from neonatal BALB/c (Xu *et al.*, 1995) and A/J epidermis (Kodali *et al.*, 2004), respectively, that are dendritic in nature, capable of antigen presentation, and have many phenotypic characteristics of LC (Takashima *et al.*, 1995; Xu *et al.*, 1995). XS52 cells were grown in CM with the addition of 2 ng/ml murine rGM-CSF (Chemicon International, Temecula, CA), 10% NS cell supernatant (supernatant conditioned by a fibroblast-like cell line, known to support the growth of epidermal APC-derived cell lines (Pierce *et al.*, 1997), and 5×10^{-5} M 2-ME (Sigma-Aldrich, St Louis, MO) at 37°C in a humidified incubator with 5% CO_2.

The HDK-1 cell line, a KLH-specific, I-A^d-restricted T helper 1 clone, was also provided by A Takashima. It was maintained in CM supplemented with 5×10^{-5} M 2-ME and 200 U/ml murine IL-2 (Chemicon Internation).

Preparation of purified epidermal LC

A preparation of LC enriched to approximately 95% homogeneity was prepared as previously described (Seiffert *et al.*, 2002). Briefly, truncal skins of mice were shaved with an electric clipper and remaining hairs were depleted with sodium thioglycolate (Nair, Carter-Wallace, New York, NY). The subcutaneous fat and carnosus panniculus were removed by blunt dissection. The skins were then floated dermis-side down for 45 minutes in Ca^{+2}/Mg^{2+}-free PBS containing 0.5 U of dispase/ml (Boehringer Mannheim, Indianapolis, IN) and 0.38% trypsin (Sigma-Aldrich). Epidermal sheets were removed with fine forceps and washed in PBS three times to remove any dermal cell contamination. Epidermal cells were dissociated by continuous mild agitation for 20 minutes in HBSS (Mediatech) supplemented with 2% fetal calf serum. The epidermal cells were then filtered through a 40-μm nylon gauge (BD Biosciences, Franklin Lakes, NJ) and washed twice in CM.

To prepare pLC, epidermal cells were incubated in a 1/2,000 dilution of Thy-1.2 Ab (Sigma-Aldrich) for 30 minutes at 4°C. The cells were then washed twice and incubated in a 1/40 dilution of low toxicity H-rabbit complement (Cederlane Laboratories, Hornby, Canada) for 30 minutes at 37°C. The cells were then washed twice in PBS and incubated in PBS with 80 μg/ml DNase I (Sigma-Aldrich) and 0.05% trypsin for 4 minutes at room temperature. The cells were finally washed in CM (this procedure enriches for LC content by selectively removing epidermal T cells and some keratinocyte), and were incubated with anti I-A^d monoclonal Ab (BD PharMingen, San Jose, CA) at a 1/50 dilution for 30 minutes at 4°C with gentle agitation. They were then incubated with goat anti-mouse IgG conjugated to magnetic microspheres (Dynabeads M-450; Dynal Biotech, Lake Success, NY) for 10 minutes with continuous gentle agitation. The pLC were obtained by repeatedly washing (up to six times) using a magnetic particle concentrator (Dynal Biotech, Lake Success, NY). By FACS analysis using anti-I-A^d mAb, this procedure yields a cell population of 92–98% LC (pLC).

Preparation of nuclear extracts

XS52 cells were plated at a density of 5×10^6 cells/plate in 25 cm^2 tissue culture dishes, cultured in CM for 24 hours, then stimulated with or without LPS in the presence or absence of NP for 3 hours and washed twice with ice-cold PBS. Cell pellets were homogenized with 400 μl of cell lysis buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 5 mM NaF, 1 mM Na_3VO_4, and 1 mM NaN_3). After a 10 minute ice bath, Nonidet P-40 was added to a final concentration of 0.5% and nuclei were isolated by centrifugation at $10{,}000 \times g$ for 1 minutes. Pelleted nuclei were lysed by incubation for 30 minutes on ice in 50 μl of nuclear lysis buffer (20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9, 420 mM NaCl, 0.2 mM EDTA, 0.2 mM EGTA, 1.5 mM MgCl, 40 mM KCl, 25% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl, and 1 μg/ml lepeptin) with agitation. Supernatants containing nulear protein were harvested by centrifugation for 15 minutes at $12{,}000 \times g$ at 4°C, the protein concentration was determined and aliquots were stored at −80°C.

Electrophoretic mobility shift assay

A double-stranded oligonucleotide containing an NF-κB binding site derived from the nucleotides −85 to −76 of the murine inducible nitric oxide synthase promoter was prepared as described (Xie *et al.*, 1994) and was kindly provided by A Ding (Weill Medical College of Cornell University, New York, NY). The sequence is as follows (NF-κB binding site is underlined):

5′-gaagctTGGGGACTCTCCCTTTG
ACCCCTGAGAGGGAAACCCTT

The probe was labeled by Klenow polymerase using [α^{32}P] dCTP to a specific activity of $0.3–1.0 \times 10^6$ c.p.m./ng. Five microgram of nuclear extracts from each test were incubated with the labeled oligonucleotide probe ($2–4 \times 10^4$ c.p.m.) in 15 μl of reaction mixture containing 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH 7.9), 1 mM EDTA, 60 mM KCL, 12% glycerol, 1 mM dithiothreitol, 2 μg poly (dI–dC) at room temperature for 20 minutes. Then, the samples were loaded onto 4.8% nondenaturing polyacrylamide gel and electrophoresed in Tris-borate-EDTA buffer (45 mM Tris-HCl, pH 8.4, 1 mM EDTA, 45 mM boric Acid) at 4°C, followed by drying of the gel and autoradiography. In competition and Ab supershift experiments, nuclear extracts were incubated for 15 minutes at room temperature with 1 μg of anti-rabbit polyclonal anti-p50 and 1 μg of anti-rabbit polyclonal anti-p65 (Santa Cruz Biotechnology) before the addition of the labeled probe.

Detection of IκBα and P-IKKα/β protein

P-IKKα/β and IκBα were detected by Western blotting with Abs specific to P-IKKα/β and IκBα. Two million XS52 cells per well in a six-well plate were cultured in CM for 24 hours, and then stimulated with or without LPS in the absence or presence of NP for 30 seconds. Fresh pLC were cultured in CM with 10 ng/ml of mGM-CSF for 1 hour, and then stimulated with or without LPS in absence or presence of NP for 30 seconds. After washing two times with cold PBS, both of XS52 cells and pLC in each well were lysed by adding 250 μl of cell lysis buffer (20 mM Tris-HCL, pH 7.5; 150 mM NaCl; 1 mM Na_2EDTA; 1 mM EGTA; 1% Triton; 2.5 mM sodium pyrophosphate; 1 mM glycerophosphate; 1 mM $NaVO_4$; 1 μg/ml lepeptin, 1 mM phenylmethylsulfonylfluoride). Cells were then sonicated for 15 seconds and microcentrifuged for 5 minutes. The protein concentration in each sample was quantitative by the bicinchoninic acid assay. Twenty-five micrograms of cell lysates were separated on a 7.5% SDS-PAGE gel and then electrotransfered to a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline buffer with 0.1% Tween-20 for 1 hour and then incubated with 1:1,000 diluted of rabbit anti-mouse P-IKKα/β and IκBα Ab (Cell Signaling, Beverly, MA) overnight at 4°C. The membrane was washed three times and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3,000 dilution) for 1 hour, and proteins were detected by a chemiluminescence detection system (Amersham, Piscataway, NJ).

Densitometry

The relative density of bands in Western blots was analyzed using a World Wide Web-based image processing system (ImageJ, http://rsb.info.nih.gov/ij/) to obtain densities in arbitrary units (AU).

To obtain the percent inhibition of NF-κB activation observed due to a NP, the following formula was used: $[1-(AU_{NP+LPS}-AU_{NP})/(AU_{LPS}-AU_{MED})] \times 100$ where AU_{NP+LPS} is the value of a band resulting from treatment with the NP and LPS, AU_{NP} is the value from treatment with the NP alone, AU_{LPS} is the value from treatment with LPS alone, and AU_{MED} is the value from treatment with medium alone.

To obtain the percent of IκBα degradation observed in response to LPS with and without the presence of NP, the following formula was used. For Figure 1b: $[1-(A_1/K_1 \div A_2/K_2)] \times 100$ where A_1 is the density of a band for IκBα after treatment at each time point, K_1 is the density of the corresponding band for glyceraldehyde-3-phosphate dehydrogenase at each time point, A_2 is the density of the band for LPS stimulation at time 0, and K_2 is the corresponding band for glyceraldehyde-3-phosphate dehydrogenase at time 0. For Figure 1c: $[1-(A_1/K_1 \div A_2/K_2)] \times 100$ where A_1 is the density of a band for IκBα after treatment, K_1 is the density of the corresponding band for IKKβ after treatment with LPS plus a NP, A_2 is the density of the band without LPS stimulation, and K_2 is the corresponding band for IKKβ without LPS stimulation.

To obtain the percent inhibition of P-IKKβ observed due to a NP, the following formula was used. For Figures 2a and 3: $[1-(P_1/K_1-P_2/K_2) \div (P_3/K_3-P_2/K_2)] \times 100$ where P_1 is the density of a band for P-IKKβ after treatment at each concentration of NP, K_1 is the density of the corresponding band for IKKβ, P_2 is the density of the band without stimulation, and K_2 is the corresponding band for IKKβ, P_3 is the density of the band for P-IKKβ after treatment with LPS but no NP and K_3 is the corresponding band for IKKβ. For Figure 2b: $[1-(P_1/K_1-P_2/K_2) \div (P_3/K_3-P_2/K_2)] \times 100$ where P_1 is the density of a band for P-IKKβ after treatment with LPS and a NP, K_1 is the density of the corresponding band for IKKβ, P_2 is the density of the band without LPS stimulation, and K_2 is the corresponding band for IKKβ, P_3 is the density of the band for P-IKKβ after treatment with LPS but no NP and K_3 is the corresponding band for IKKβ.

Cytokine determination

The TNFα level in supernatants was detected by using murine TNFα, IL-12 p40, and IL-1β sandwich ELISA kits (R&D systems, Minneapolis, MN) following the manufacturer's instructions. IFNγ production by HDK-1 cells was analyzed by OptEIA sets of capture Ab and biotinylated detection mAb for murine IFN-γ (BD Pharmingen). One hundred microliters of capture Ab (4 μg/ml in coating buffer) was added to each well in 96-well, flat-bottom plates and incubated overnight at 4°C. Then each well was aspirated and washed three times with PBS-Tween 20 (0.05%). Wells were blocked with 200 μl of 10% fetal bovine serum in PBS, followed by aspirating and washing each well three times with PBS-Tween 20 (0.05%). One hundred microliters of each standard and sample were then added to wells. After incubation at room temperature for 2 hours, each well was aspirated and washed five times with PBS-Tween 20 (0.05%). Then 100 μl of detection Ab (1 μg/ml) was added to each well, followed by incubation at room temperature for 1 hour. Wells were aspirated and washed five times with PBS-Tween 20 (0.05%). Then, 100 μl of avidin-horseradish peroxidase (1/2,000) was added to each well, followed by incubation for 30 minutes at room temperature. After aspiration and washing of each well seven times with PBS-Tween 20 (0.05%), 100 μl of substrate solution (tetramethylbenzidine and hydrogen peroxide) was added to each well at room temperature in the dark. Fifty microliters of 1 M H_3PO_4 was added to each well to stop the reaction, and wells were read at 450 nm.

Statistical analysis

The significance of differences among groups was measured by Students' two-tailed *t*-test for unpaired samples (Excel software; Microsoft, Seattle, WA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported by NIH grant 5R01 AR042429 (RDG and JW), a gift from the Jacob L and Lillian Holtzmann Foundation, a grant from the Edith C Blum Foundation, the Carl and Fay Simons Family Trust, the Oliver and Elizabeth Stanton Foundation, contributions from the Ann L and Herbert J Siegel Philanthropic Fund, contributions from Mr and Mrs Blair O'Connor, and a gift from Call on an Angel.

REFERENCES

Ardeshna KM, Pizzey AR, Devereux S, Khwaja A (2000) The PI3 kinase, p38 SAP kinase, and NF-kappaB signal transduction pathways are involved in the survival and maturation of lipopolysaccharide-stimulated human monocyte-derived dendritic cells. *Blood* 96:1039–46

Asahina A, Hosoi J, Beissert S, Stratigos A, Granstein RD (1995) Inhibition of the induction of delayed-type and contact hypersensitivity by calcitonin gene-related peptide. *J Immunol* 154:3056–61

Bennett CL, van Rijn E, Jung S, Inaba K, Steinman RM, Kapsenberg ML *et al.* (2005) Inducible ablation of mouse Langerhans cells diminishes but fails to abrogate contact hypersensitivity. *J Cell Biol* 169:569–76

Brain SD, Williams TJ (1988) Neuropharmacology of peptides in skin. *Semin Dermatol* 7:278–83

Caux C, Dezutter-Dambuyant C, Schmitt D, Banchereau J (1992) GM-CSF and TNF-alpha cooperate in the generation of dendritic Langerhans cells. *Nature* 360:258–61

Cumberbatch M, Bhushan M, Dearman RJ, Kimber I, Griffiths CE (2003) IL-1beta-induced Langerhans' cell migration and TNF-alpha production in human skin: regulation by lactoferrin. *Clin Exp Immunol* 132:352–9

Cumberbatch M, Griffiths CE, Tucker SC, Dearman RJ, Kimber I (1999) Tumour necrosis factor-alpha induces Langerhans cell migration in humans. *Br J Dermatol* 141:192–200

de Saint-Vis B, Fugier-Vivier I, Massacrier C, Gaillard C, Vanbervliet B, Ait-Yahia S *et al.* (1998) The cytokine profile expressed by human dendritic cells is dependent on cell subtype and mode of activation. *J Immunol* 160:1666–76

Delgado M, Munoz-Elias EJ, Martinez C, Gomariz RP, Ganea D (1999) VIP and PACAP38 modulate cytokine and nitric oxide production in peritoneal macrophages and macrophage cell lines. *Ann NY Acad Sci* 897:401–14

Feldmann M, Andreakos E, Smith C, Bondeson J, Yoshimura S, Kiriakidis S *et al.* (2002) Is NF-kappaB a useful therapeutic target in rheumatoid arthritis? *Ann Rheum Dis* 61(Suppl. 2):ii13–8

Fujita H, Asahina A, Tada Y, Fujiwara H, Tamaki K (2005) Type I interferons inhibit maturation and activation of mouse Langerhans cells. *J Invest Dermatol* 125:126–33

Ghosh S, May MJ, Kopp EB (1998) NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses. *Ann Rev Immunol* 16:225–60

Grabbe S, Bruvers S, Granstein RD (1992) Effects of immunomodulatory cytokines on the presentation of tumor-associated antigens by epidermal Langerhans cells. *J Invest Dermatol* 99:66S–8S

Hosoi J, Murphy GF, Egan CL, Lerner EA, Grabbe S, Asahina A *et al.* (1993) Regulation of Langerhans cell function by nerves containing calcitonin gene-related peptide. *Nature* 363:159–63

Jeon YJ, Han SH, Kang JS, Koh WS, Yang KH (1999) Acetylaminofluorene inhibits nitric oxide production in LPS-stimulated RAW 264.7 cells by blocking NF-kappa B/Rel activation. *Toxicol Lett* 104:195–202

Kaplan DH, Jenison MC, Saeland S, Shlomchik WD, Shlomchik MJ (2005) Epidermal Langerhans cell-deficient mice develop enhanced contact hypersensitivity. *Immunity* 23:611–20

Kissenpfennig A, Henri S, Dubois B, Laplace-Builhe C, Perrin P, Romani N *et al.* (2005) Dynamics and function of Langerhans cells *in vivo*: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells. *Immunity* 22:643–54

Kodali S, Ding W, Huang J, Seiffert K, Wagner JA, Granstein RD (2004) Vasoactive intestinal peptide modulates Langerhans cell immune function. *J Immunol* 173:6082–8

Kodali S, Friedman I, Ding W, Seiffert K, Wagner JA, Granstein RD (2003) Pituitary adenylate cyclase-activating polypeptide inhibits cutaneous immune function. *Eur J Immunol* 33:3070–9

Laburthe M, Couvineau A, Marie JC (2002) VPAC receptors for VIP and PACAP. *Receptors Channels* 8:137–53

Li Q, Verma IM (2002) NF-kappaB regulation in the immune system. *Nat Rev Immunol* 2:725

Mabuchi S, Ohmichi M, Nishio Y, Hayasaka T, Kimura A, Ohta T *et al.* (2004) Inhibition of NFkappaB increases the efficacy of cisplatin in *in vitro* and *in vivo* ovarian cancer models. *J Biol Chem* 279:23477–85

Mulder H, Jongsma H, Zhang Y, Gebre-Medhin S, Sundler F, Danielsen N (1999) Pituitary adenylate cyclase-activating polypeptide and islet amyloid polypeptide in primary sensory neurons: functional implications from plasticity in expression on nerve injury and inflammation. *Mol Neurobiol* 19:229–53

Odum L, Petersen LJ, Skov PS, Ebskov LB (1998) Pituitary adenylate cyclase activating polypeptide (PACAP) is localized in human dermal neurons and causes histamine release from skin mast cells. *Inflamm Res* 47:488–92

Pettit AR, Quinn C, MacDonald KP, Cavanagh LL, Thomas G, Townsend W *et al.* (1997) Nuclear localization of RelB is associated with effective antigen-presenting cell function. *J Immunol* 159:3681–91

Pierce JW, Schoenleber R, Jesmok G, Best J, Moore SA, Collins T *et al.* (1997) Novel inhibitors of cytokine-induced IkappaBalpha phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects *in vivo*. *J Biol Chem* 272:21096–103

Poligone B, Weaver DJ Jr, Sen P, Baldwin AS Jr, Tisch R (2002) Elevated NF-kappaB activation in nonobese diabetic mouse dendritic cells results in enhanced APC function. *J Immunol* 168:188–96

Rescigno M, Martino M, Sutherland CL, Gold MR, Ricciardi-Castagnoli P (1998) Dendritic cell survival and maturation are regulated by different signaling pathways. *J Exp Med* 188:2175–80

Schmitt D (1999) Immune functions of the human skin. Models of *in vitro* studies using Langerhans cells. *Cell Biol Toxicol* 15:41–5

Schreiber S, Kilgus O, Payer E, Kutil R, Elbe A, Mueller C *et al.* (1992) Cytokine pattern of Langerhans cells isolated from murine epidermal cell cultures. *J Immunol* 149:3524–34

Schulze E, Witt M, Fink T, Hofer A, Funk RH (1997) Immunohistochemical detection of human skin nerve fibers. *Acta Histochem* 99:301–9

Seiffert K, Hosoi J, Torii H, Ozawa H, Ding W, Campton K (2002) Catecholamines inhibit the antigen-presenting capability of epidermal Langerhans cells. *J Immunol* 168:6128–35

Sen P, Bhattacharyya S, Wallet M, Wong CP, Poligone B, Sen M *et al.* (2003) NF-kappa B hyperactivation has differential effects on the APC function of nonobese diabetic mouse macrophages. *J Immunol* 170:1770–80

Sherwood NM, Krueckl SL, McRory JE (2000) The origin and function of the pituitary adenylate cyclase-activating polypeptide (PACAP)/glucagon superfamily. *Endocr Rev* 21:619–700

Steinman R, Hoffman L, Pope M (1995) Maturation and migration of cutaneous dendritic cells. *J Invest Dermatol* 105:2S–7S

Takashima A, Edelbaum D, Kitajima T, Shadduck RK, Gilmore GL, Xu S *et al.* (1995) Colony-stimulating factor-1 secreted by fibroblasts promotes the growth of dendritic cell lines (XS series) derived from murine epidermis. *J Immunol* 154:5128–35

Thomas PG, Carter MR, Da'dara AA, DeSimone TM, Harn DA (2005) A helminth glycan induces APC maturation via alternative NF-kappa B activation independent of I kappa B alpha degradation. *J Immunol* 175:2082–90

Torii H, Hosoi J, Asahina A, Granstein RD (1997) Calcitonin gene-related peptide and Langerhans cell function. *J Investig Dermatol Symp Proc* 2:82–6

Trede NS, Tsytsykova AV, Chatila T, Goldfeld AE, Geha RS (1995) Transcriptional activation of the human TNF-alpha promoter by superantigen in human monocytic cells: role of NF-kappa B. *J Immunol* 155:902–8

Viatour P, Merville MP, Bours V, Chariot A (2005) Phosphorylation of NF-kappaB and IkappaB proteins: implications in cancer and inflammation. *Trends Biochem Sci* 30:43–52

Wang B, Fujisawa H, Zhuang L, Kondo S, Shivji GM, Kim CS *et al.* (1997) Depressed Langerhans cell migration and reduced contact hypersensitivity response in mice lacking TNF receptor p75. *J Immunol* 159:6148–55

Xie QW, Kashiwabara Y, Nathan C (1994) Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. *J Biol Chem* 269:4705–8

Xu S, Ariizumi K, Edelbaum D, Bergstresser PR, Takashima A (1995) Cytokine-dependent regulation of growth and maturation in murine epidermal dendritic cell lines. *Eur J Immunol* 25:1018–24

Yao J, Mackman N, Edgington TS, Fan ST (1997) Lipopolysaccharide induction of the tumor necrosis factor-alpha promoter in human monocytic cells. Regulation by Egr-1, c-Jun, and NF-kappaB transcription factors. *J Biol Chem* 272:17795–801

Zaidi M, Moonga BS, Bevis PJ, Bascal ZA, Breimer LH (1990) The calcitonin gene peptides: biology and clinical relevance. *Crit Rev Clin Lab Sci* 28:109–74